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Dendritic cells (DC) are an integral part of the immune system's response to cancer. A number of clinical trials have been initiated to use these cells in the treatment of melanoma, prostate cancer, and lymphoma. In preparation for treating breast cancer patients with DC, this proposal examines a fundamental issue that needs to be resolved before proceeding with this exciting new therapy. We hypothesized that the processing and presentation of multiple tumor antigen epitopes by DC is a more efficient and effective way of stimulating T cell responses than current HLA-restricted peptide-based methods. The goal of this proposal is to develop practical methods by which immune cells from patients with breast cancer can be used to promote effective anti-tumor responses. In the past year, we have been evaluating the phenotype and functional activity of breast cancer patients' DC in preparation for their use in tumor antigen-presentation. We also have developed a rapid method to assess patient's T cell function prior to treatment. In addition, we have optimized testing procedures to evaluate anti-tumor cytotoxicity. This progress is consistent with the proposal's Statement of Work, and leaves us well-positioned to achieve the next goals of the proposal.

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FOREWORD

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N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Stephen M. Kwiatkowski 7-27-00

PI - Signature Date

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INTRODUCTION

Dendritic cells (DC) are an integral part of the immune systems' response to cancer. When loaded with tumor antigens, DC have great value as immunotherapeutic agents. However, the method for arming the DC with antigens which results in the most effective immune response has yet to be defined. We theorized that the broadest possible mix of tumor antigens might provide the best material for stimulating an effective immune response. We hypothesized that the processing and presentation of multiple tumor antigen epitopes by DC would be the most efficient and effective way of stimulating T cell responses. The goal of this proposal is to develop practical methods by which immune cells from patients with breast cancer can be used to promote effective anti-tumor responses. In this study we will compare multiple methods of arming DC with tumor antigens including: 1) purified immunodominant peptides which are specific for a single antigen and a single Class I MHC molecule, 2) transduced cDNA encoding for a single tumor antigen which will allow the recipient DC to intrinsically process and present all possible antigenic peptides (immunodominant and sub-dominant) within the context of all available MHC molecules, and 3) extracts from autologous whole tumor cells which will provide a broad mix of tumor antigens (both defined and undefined antigens) for processing and presentation. The information obtained from this study will further our understanding of the interactions between DC and T cells which lead to the generation of tumor-antigen-specific responses. This understanding will be valuable in the development of immunotherapeutic treatments for breast cancer.

ANNUAL SUMMARY

RESEARCH ACCOMPLISHMENTS:

For the year 7-1-99 through 6-30-00 (proposal months 13-24), we are pleased to report progress on Tasks 1-3 in the proposal Statement of Work. This progress is composed of advances in three main research areas: patient recruitment, evaluation of patient DC, and development of assays to assess anti-tumor reactivity.

Task 1. To identify HLA-A2$^+$ breast cancer patients whose tumors do and do not overexpress Her-2/neu. (Scheduled for months 1-36).

Patient Recruitment

The UCLA IRB has approved the recruitment flier and informed consent forms for the proposal. The process of patient recruitment and enrollment is underway.

Evaluation of Patient Dendritic Cells

Research by ourselves and others has demonstrated that the tumor environment has an immunosuppressive effect on DC. Many of these studies have been done on pathology specimens in a retrospective fashion. Studies determining the immune status of patients' DC in a prospective manner are needed. In the past year, we have begun to characterize the phenotype and functional activity of the breast cancer patients' DC. The original proposal called for the patients' monocyte-derived dendritic cells (DC) to be evaluated for antigen-presenting cell phenotype and T cell stimulatory activity. Since the proposal was written, antibodies and procedures have been developed to also measure the rare DC which are present in peripheral blood. These circulating DC are composed of both CD11c$^+$ and CD11c$^-$ populations, and are defined by their lack of expression of lymphocyte and monocyte lineage antigens. Previously, the identification of these populations could only be made following extensive purification. The new procedure allows these cells to be identified directly from peripheral blood, without manipulation. The cells are stained with a cocktail of antibodies to lineage markers, as well as anti-HLA-DR and CD11c. An example of this staining is shown in Figure 1.

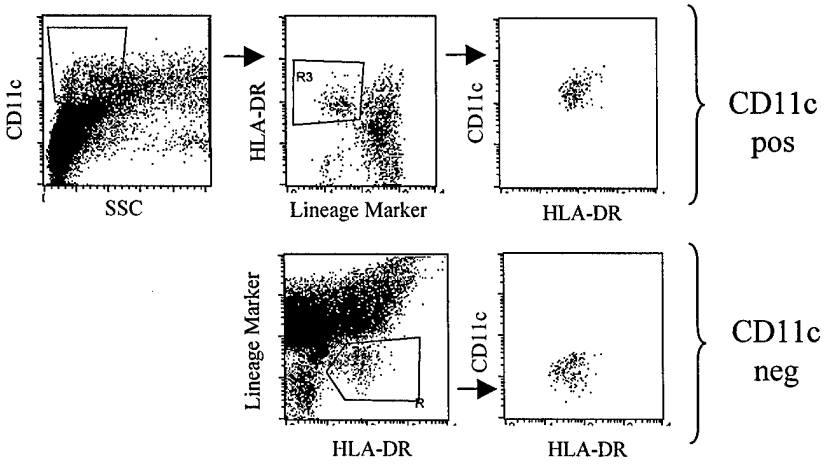

Figure 1. Identification of CD11c positive and negative circulating DC by FACS analysis and sequential gating.

In the evaluations of patients' monocyte-derived DC performed thus far, we have found defects in expression of antigen-presenting cell and costimulatory molecules in some of the patients (e.g. CD86 expressed at 42% of the level seen on DC from control subjects), as well as over-expression of the monocyte marker CD14 (3 to 4 times higher on patients' cells). CD14 is normally down-regulated during the process of DC maturation, and over-expression may correspond to defects in

function. As a corollary to these studies, we are investigating ways to enhance the phenotype and function of the patient's DC by either adding stimulatory cytokines or blocking inhibitory cytokines during the maturation process. These approaches may be necessary to optimize the ability of patient's DC to function as a vaccine in future clinical studies.

Our goal is to eventually use antigen-armed DC in clinical trials, and the success of these treatments will be dependent on T cell, as well as DC, function. Because immunotherapeutic approaches to cancer therapy rely on the ability of patients' T cells to respond to a cancer vaccine, a rapid method to determine their immune status is needed. We have developed an intracellular cytokine staining method which measures the production of interferon-γ by mitogen-stimulated T cells. In preliminary experiments, we have seen a reduction in the ability of patients' T cells to produce interferon-γ (see Figure 2). A reduction in the proportion of responding cells could limit the patients ability to mount an immune response. The patient's $CD3^+$ T cells also expressed less of the activation marker CD69, although their expression of the IL-2 receptor CD25 was similar to the controls.

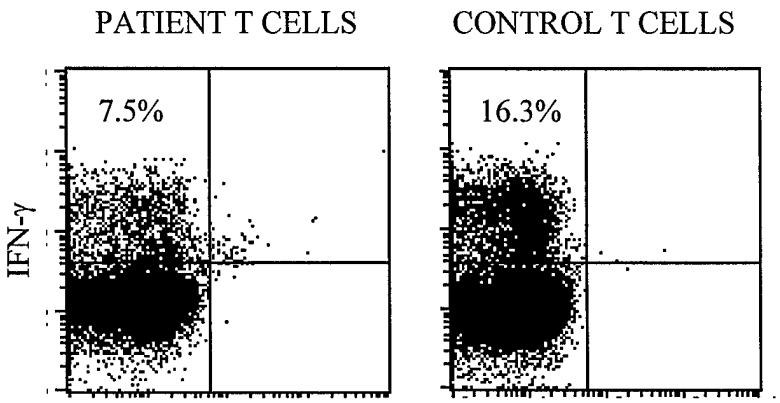

Figure 2. The percentage of IFN-γ-producing cells is reduced in breast cancer patients. Control responses ranged from 15-25%.

This assay could potentially be used to screen patients prior to immunotherapy to determine which are most likely to respond. Many immunotherapeutic approaches have a limited response rate, and the ability to determine responders pre-treatment would spare patients unnecessary clinic visits and procedures. Additional data regarding the range of cytokine production in patients vs. controls are essential before applying this method as a screening tool.

Task 2. To obtain peripheral blood and tumor specimens from these patients and use them to generate DC, isolate T cells and produce tumor cell lysates. (Scheduled for months 2-40).

Tumor Lysate Preparation and Use:

We have modified the protocol for disrupting the tumor cells to produce antigens in a form usable by DC. Previously, we had found that sonication resulted in higher protein concentrations than multiple freeze-thaw cycles. However, subsequent investigations by others have found that the larger fragments generated by freeze/thaw may be better taken up by the DC, and will result in better stimulation of the tumor-specific T cells. In addition, we have changed the pulsing procedure to use the lysate on a tumor cell equivalent:DC ratio, rather than a protein concentration/DC basis. The elimination of the protein determination simplifies the procedure and will make it more practical for clinical use. Based on these experiments, we have modified the protocol for tumor lysate preparation and DC-pulsing as follows: (1) tumor samples are treated with enzymes to produce a single cell suspension, (2) lymphocytes are removed with anti-CD45, (3) the remaining tumor cells are disrupted by freeze/thaw, (4), and the lysate is frozen in aliquots for future use. When needed for the assays, the lysates will be thawed and used to arm DC with antigens by co-incubation for 2-4 hours.

Task 3. To determine the frequency of Her-2-specific T cells generated using the three different antigen-arming methods with a modified limiting dilution procedure. (Scheduled for months 2-40).

Assay Development

Assays to monitor the generation of Her-2-specific T cell responses are essential to both this proposal (used in Tasks 3 and 4), and future clinical trials. In preparation for these studies, we have refined the alamar blue assay for the assessment of tumor cytotoxicity. The alamar blue assay uses a non-toxic metabolic indicator of viable cells that fluoresces upon mitochondrial reduction. Viable cells exhibit a measurable level of this activity, and reductions in this level indicate cell death. We have shown previously that this assay measures cytotoxicity as effectively as the ^{51}Cr-release assay, and is, in fact, more sensitive at lower Effector:Target cell ratios.

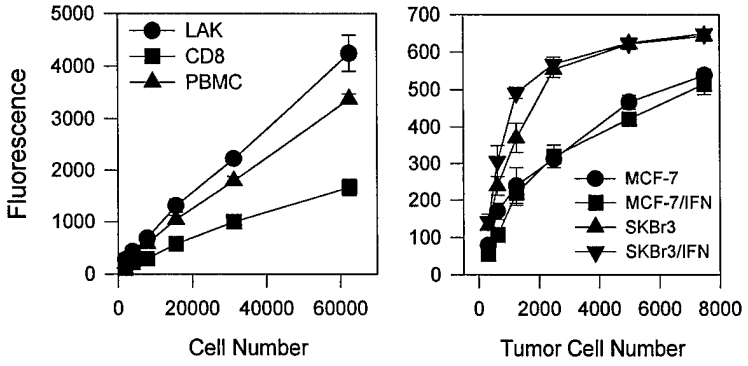

Figure 3. Alamar blue fluorescence is proportional to both effector cell and tumor cell number.

However, we have found some limitations to this assay as originally designed. In order for results to be valid, the number of tumor cells per well must be in the linear portion of the cell number vs. fluorescence curve (Figure 3). In addition, since cytotoxicity is determined by a loss of viability and depends on the total signal of tumors alone plus effector alone being additive, the total signal must not exceed the maximal fluorescence detectable by the plate reader. Each tumor is unique in its fluorescence level, so a simple titration assay measuring various numbers of tumor cells must be performed before using the tumor cells as targets in a cytotoxicity assay. This step will ensure that the levels of cytotoxicity measured in subsequent assays are accurate.

TRAINING ACCOMPLISHMENTS:

In the past year, I received a promotion to Assistant Adjunct Professor (effective 10/01/99). This is an acknowledgement of both my research efforts, and my development as an independent investigator. With this position, I become eligible for membership in the UCLA Jonsson Comprehensive Cancer Center. Privileges of membership include access to financial support and office and research space controlled by the Cancer Center, as well as priority access to shared resources and core services. My membership application is being reviewed.

The expertise in immune assay development which I have gained from this supported work has led to interactions with oncologists who are interested in utilizing these methods in clinical trial monitoring. In turn, these interactions have provided valuable experience in clinical trial procedures and regulatory issues which will enable me to better implement the translational aspects of this project.

In summary, the progress in the past year is consistent with the proposal Statement of Work, and leaves us well-positioned to achieve the next goals of the proposal.

KEY RESEARCH ACCOMPLISHMENTS

- Renewed IRB approval for recruitment fliers and informed consent forms
- Initiated enrollment of patients for *ex vivo* studies
- Began assessment of phenotypic and functional activity of patient's circulating and monocyte-derived DC
- Developed method to evaluate functional status of patients' T cells
- Optimized Alamar Blue assay for the measurement of tumor cytotoxicity
- Modified method for preparing and using tumor lysate
- Initiated construction of Her2-expressing adenoviral vector

REPORTABLE OUTCOMES

1. Promotion from Assistant Researcher to Assistant Adj. Professor (effective 10/01/99).
2. Membership in UCLA Jonsson Comprehensive Cancer Center is pending.
3. Presented abstract at Era of Hope Meeting, June 8-11, Atlanta, GA.

ASSESSING ANTI-HER-2 IMMUNE RESPONSES INDUCED BY ANTIGEN-PULSED DENDRITIC CELLS

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Dendritic cells (DC) are an integral part of the immune systems' response to cancer. When loaded with tumor antigens, DC have promising potential as immunotherapeutic agents. However, the method for arming the DC with antigens that results in the most effective immune response has yet to be defined. We hypothesized that the processing and presentation of multiple tumor antigen epitopes by DC would be the most efficient and effective way of stimulating T cell responses.

This hypothesis will be tested by comparing multiple methods of arming breast cancer patients' DC with tumor antigens, including 1) purified immunodominant peptides (specific for the HER-2 antigen and a single Class I MHC molecule), 2) transduced cDNA encoding for the HER-2 tumor antigen (allowing the processing and presentation of multiple antigenic peptides in the context of all available MHC molecules), and 3) extracts from autologous whole tumor cells (providing a broad mix of both defined and undefined tumor antigens).

In our results to date, we have optimized procedures to assess anti-tumor cytotoxicity and tumor antigen-specific cytokine release by T cells. We concluded that the AlamarBlue assay for cytotoxicity was both more sensitive than the standard ^{51}Cr-release method, and more accurate for assessing cytotoxicity against autologous tumor. Also, we determined optimal conditions for the ELISpot assay, which measures the frequency of cytokine-producing T cells. These assays are essential in evaluating the various DC arming methods, and will be valuable in monitoring the efficacy of future clinical trials. In addition, we have evaluated methods for preparing tumor lysate and whole tumor antigen and devised a strategy for expressing the HER-2 gene in DC.

The goal of this proposal is to develop practical methods by which immune cells from patients with breast cancer can be used to promote effective anti-tumor responses. The development of immunological assays to determine the efficacy of those methods is an important first step in the achievement of this goal.

The U.S. Army Medical Research and Materiel Command under DAMD17-98-1-8181 supported this work.